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# Noncoding Flavivirus RNA Displays RNA Interference Suppressor Activity in Insect and Mammalian Cells

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West Nile virus (WNV) and dengue virus (DENV) are highly pathogenic, mosquito-borne flaviviruses (family *Flaviviridae*) that cause severe disease and death in humans. WNV and DENV actively replicate in mosquitoes and human hosts and thus encounter different host immune responses. RNA interference (RNAi) is the predominant antiviral response against invading RNA viruses in insects and plants. As a countermeasure, plant and insect RNA viruses encode RNA silencing suppressor (RSS) proteins to block the generation/activity of small interfering RNA (siRNA). Enhanced flavivirus replication in mosquitoes depleted for RNAi factors suggests an important biological role for RNAi in restricting virus replication, but it has remained unclear whether or not flaviviruses counteract RNAi via expression of an RSS. First, we established that flaviviral RNA replication suppressed siRNA-induced gene silencing in WNV and DENV replicon-expressing cells. Next, we showed that none of the WNV encoded proteins displayed RSS activity in mammalian and insect cells and in plants by using robust RNAi suppressor assays. In contrast, we found that the 3′-untranslated region-derived RNA molecule known as subgenomic flavivirus RNA (sfRNA) efficiently suppressed siRNA- and miRNA-induced RNAi pathways in both mammalian and insect cells. We also showed that WNV sfRNA inhibits *in vitro* cleavage of double-stranded RNA by Dicer. The results of the present study suggest a novel role for sfRNA, i.e., as a nucleic acid-based regulator of RNAi pathways, a strategy that may be conserved among flaviviruses.

Arthropod-borne (arbo)viruses form a diverse group of medically important viruses, many of which are emerging pathogens (72). Arboviruses take a unique position within the virosphere by displaying active replication in both vertebrate hosts (humans, other mammals, and birds) and invertebrate vectors (e.g., mosquitoes, ticks, midges, and sandflies) (41). Upon infection of mosquitoes, the virus persistently replicates in multiple tissues of the insect, and high virus titers accumulate in the salivary glands by the end of this so-called extrinsic incubation time, typically 1 to 2 weeks. Since the virus needs the vector for successful infection of the vertebrate hosts to complete the transmission cycle, evolutionary pressure has likely caused the virus to be only mildly or nonpathogenic to the arthropod host (17). Nonetheless, to perpetuate the viral life cycle involving invertebrate vector and vertebrate host, the virus must be sufficiently equipped to cross the initial midgut infection barrier in the vector and must be able to disseminate within the arthropod to eventually accumulate progeny virus in the salivary glands.

Mosquitoes and other arthropods have an array of mechanisms to fight microbial and viral infections. RNA-induced gene silencing or RNA interference (RNAi) is the key component of the insect innate immune system to limit a diverse range of RNA viruses, including flaviviruses (6, 55), while the Toll, IMD, and JAK-STAT pathways also contribute to control flavivirus infection in mosquitoes (17). As a countermeasure, insect-specific viruses have been demonstrated to suppress this antiviral RNAi response by producing specialized proteins that obstruct one or more of the key RNAi components. Well-studied examples are the Flock House virus (FHV) B2 viral RNA silencing suppressor (RSS) (37), the Cricket paralysis virus (CrPV) 1A RSS (44, 71), and the related *Drosophila* C virus (DCV) 1A protein (65). For FHV, it was shown that suppression of antiviral RNAi by expression of a viral

RSS was crucial for establishing efficient viral replication and virion production (31).

Until now, no viral RSS have conclusively been identified in arboviral genomes. For dengue virus (DENV), it was suggested from preliminary experiments that none of the DENV mature viral proteins could suppress RNAi (32). More recently, it was hypothesized that arboviruses may not even need a RSS, since they subject themselves to antiviral RNAi and replicate at lower levels to establish persistent infection of the insect host (64). While it remains to be seen whether this is true for all arboviruses without exception, persistent virus infection of the arthropod—the hallmark of arbovirus replication—does not inevitably mean that the virus does not display RSS activity. For example, the insect-specific viruses DCV, CrPV, and FHV all encode strong RSSs in their genomes (44, 65, 71), and yet all of these viruses can persistently infect their insect hosts. Conversely, it can be hypothesized that persistently infecting arboviruses may encode RSSs, for example, to allow sufficient levels of viral replication in vector insects, especially in view of the high potency of the antiviral RNAi response (6).

West Nile virus (WNV) and DENV are highly pathogenic, mosquito-borne viruses (family *Flaviviridae*, genus *Flavivirus*) that cause severe disease and death in humans (34). Flavivirus virions contain a single copy of the viral genome, which encodes a

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polyprotein that is proteolytically cleaved into seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and three structural proteins (C, prM, and E). The positive-stranded RNA genome of ~11 kb is flanked by 5' and 3' untranslated regions (UTRs), which play essential roles in initiation of translation and RNA replication (40). Interestingly, recent reports have demonstrated that many, most likely all, flaviviruses produce a second, subgenomic flavivirus RNA (sfRNA), which accumulates to high levels in a diverse range of infected mammalian and insect cells (33, 52, 56). sfRNA is a positive-sense, noncoding RNA molecule representing the last 525 nucleotides (nt) (in the case of WNV) of the viral genomic RNA that is generated as the result of incomplete degradation of genomic RNA by the cellular exoribonuclease XRN1 in cytoplasmic processing bodies (PBs) (19, 52, 60). PBs are cytoplasmic granules functional in mRNA degradation, mRNA surveillance and translational repression. The RNAi machinery is also concentrated in PBs (16). It has been shown that sfRNA production is required for viral pathogenicity in a mammalian animal model, although its exact mode of action remains to be uncovered (52). Recent studies suggest that sfRNA plays a role in inhibiting the alpha/beta interferon (IFN- $\alpha/\beta$ ) response in mice (59) and also may serve as the main source of WNV-encoded miRNA in mosquito cells (25).

According to the arbovirus definition, flaviviruses actively replicate in both the invertebrate vector and the vertebrate host and thus encounter an array of different host immune responses, including RNAi during virus replication in mosquitoes (17). By depleting crucial insect RNAi factors it became clear that RNAi is very efficient in limiting flavivirus replication in insects (10, 55). However, it remains to be uncovered how exactly flaviviruses evade and/or suppress the antiviral RNAi response.

Here we have studied the interaction between WNV and RNAi in different model systems. We have screened the various WNV products, including viral proteins and sfRNA, for activity as RNAi suppressor in different RNAi suppressor assays. We show that sfRNA but not viral proteins display RNAi suppressor activity in insect and mammalian cells. Finally, we provide evidence using DENV that this novel role of sfRNA is characteristic for flaviviruses.

## MATERIALS AND METHODS

**Plasmids.** The mammalian expression plasmids pGL3 and pRL-CMV (Promega), the short hairpin-encoding plasmid Firefly luciferase (pShh1-Ff1) (48), or the nonspecific pEF-MBP, pEF-MBP-NS3, were as previously described (57). The 3'UTRs of WNV and DENV and MBP-HDVr were cloned into the mammalian expression vector pcDNA-DEST40 (Invitrogen) downstream of the cytomegalovirus (CMV) promoter using Gateway technology. The pAdvantage construct expressing adenovirus VA RNAI/II was purchased from Promega. The miRNA-based sensor constructs pCMV-Fluc-miRNA30-AP, pCMV-Fluc-random, pCMV-hsa-miRNA30, and pCMV-hsa-miRNA21 have been described previously (77). The selectable WNV and DENV replicons have been previously described (35, 53, 54). The insect expression vectors encoding MBP-HDVr, the DENV 3'UTR, and the WNV 3'UTR either fused to MBP or alone were cloned behind the baculovirus OpIE2 promoter into the pIB-GW (58) vector using Gateway technology. The expression vectors encoding either MBP or *Cymbidium ringspot virus* (CymRSV) P19 have been described previously, as well as the inducible insect miRNA-based sensor constructs, pMT-FF-3'UTR, pMT-pri-dme-miRNA1, and pMT-pri-dme-miRNA-12 (58) and the inducible Firefly and *Renilla* luciferase constructs (65), all expressed via the *Drosophila* metallothionein gene promoter. Insect expression vectors encoding short hairpin RNA

were constructed by annealing previously described DNA oligonucleotides (69) either against Firefly luciferase or enhanced green fluorescent protein (eGFP) and cloning these as KpnI-XbaI fragments in pMT-B (Invitrogen) behind the *Drosophila* metallothionein gene promoter. For the RNA silencing experiments in U4.4 cells, Firefly and *Renilla* luciferase constructs were used, which have previously been described (46) and are expressed via the OpIE2 or AcIE1 promoters, respectively.

To check the functionality of the WNV miRNA sensor constructs (described in reference 25 and now recloned in pGL3 [Promega] for expression in mammalian cells), pSuper plasmids expressing small interfering RNA (siRNA) from the H1 RNA polymerase III promoter were constructed (8). The following complementary oligonucleotides with (partial) restriction sites (indicated in boldface) and complementary regions (underlined) were designed according to methods published online by OligoEngine and were cloned in the BglII and HindIII sites of pSuper: pSUPER-A1A2-F, **GATCCCCGCTCTGCACAACCA**GCCACACGGCACTTCAAGAGAGTGCCGTGTGGCTGGTTGTGCAGAGCTTTTTA; pSUPER-A1A2-R, **AGCTTAAAAAGCTCTGCACAAC**CAGCCACACGGCACTCTCTTGAAGTGCCGTGTGGCTGGTTGTGCAGAGCGGG; pSUPER-C1C2-F, **GATCCCCGACAATGGTGGCTGGT**GGTGCAGAGAATTCAAGAGATTCTCGCACCAACAGCCACCATTGTCTTTTTTA; and pSUPER-C1C2-R, **AGCTTAAAAAGACAATGGTGGCT**GGTGGTGCGAGAATCTCTTGAATCTCGCACCACCAACCCATTGTCGGG. Plasmid pSuper-A1A2 expresses a short hairpin consisting of A1A2, a short loop region and the reverse complement sequence A1A2rc. Upon expression in mammalian cells, this plasmid expresses siRNA that will silence transcripts containing both A1A2 or A1A2rc sequences. Similarly, pSuper-C1C2 targets C1C2 or C1C2rc containing transcripts.

**Cell culture and transfection.** African green monkey kidney Vero and baby hamster kidney BHK-21 cells were grown as a monolayer in Dulbecco modified Eagle medium (Gibco/BRL) supplemented with 10% fetal calf serum (FCS; Gibco), streptomycin (100  $\mu$ g/ml), and penicillin (100 U/ml) at 37°C and 5% CO<sub>2</sub>. To reach a confluence of 60 to 70% at the time of transfection, the cells were trypsinized 24 h pretransfection and seeded in 24-well plates at a concentration of  $1.1 \times 10^5$  cells per well. Transfections were performed using JetPei according to the manufacturer's instructions. To create stable mammalian replicon cell lines, Vero cells were transfected with capped, *in vitro*-transcribed replicon RNA, using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. At 24 h posttransfection (hpt), transfected cells were selected with puromycin (WNV replicon [36, 53]) or G418 (DENV replicon [54]). For the transient RNA silencing suppressor assays using short hairpin constructs as inducer molecules, (replicon) cells were transfected with luciferase expression plasmids, i.e., 100 ng of Firefly luciferase (pGL3; Promega), 2 ng of *Renilla* luciferase (pRL-CMV; Promega), and 4 ng of short hairpin encoding plasmids, either nonspecific or Firefly luciferase specific (pShh1-Ff1 [48]). In the case of transient expression of the RNA silencing suppressor, the cells were also cotransfected with the corresponding expression plasmid (MBP, MBP-NS3, MBP-NS3mutant, MBP-HDVr, WNV sfRNA, or DENV sfRNA).

The miRNA-based sensor experiments were performed in a similar way as previously described for the shRNA-induced silencing experiments. Briefly, cells were seeded in 24-well plates and cotransfected with 200 ng of sensor construct (pCMV-FF-miRNA30-AP or pCMV-FF-random [77]), 2 ng of *Renilla* luciferase plasmid (pRL-CMV; Promega), and 10 ng of pri-miRNA expression plasmid vectors expressing either human pri-hsa-miRNA21 or pri-hsa-miRNA30 (77). All cells were lysed at 48 hpt, and the luciferase activity was determined using a dual luciferase reporter assay (15).

*Drosophila melanogaster* Schneider-2 (S2) cells were grown in Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated FCS (Gibco) at 28°C. The cells were seeded 24 h pretransfection in a 96-well plate at a concentration of  $5 \times 10^4$  cells per well. Transfections were performed using Cellfectin II (Invitrogen) according to the manufacturer's instructions. For the shRNA suppressor assays, the S2 cells were

cotransfected with luciferase-expressing plasmids (15 ng of pMT-Fluc and 6 ng of pMT-Rluc) and 50 ng of short hairpin-expressing vectors, either Fluc specific or nonspecific. The cells were also cotransfected with 100 ng of the RNA silencing suppressor expressing plasmid (MBP, Rice Hoja Blanca virus [RHBV] NS3, or WNV sfRNA). For the miRNA-based suppressor assays, the S2 cells were cotransfected with 100 ng (unless stated otherwise) of RNA silencing suppressor expression plasmid (MBP, *Carnation Italian ringspot virus* [CIRV] P19, or WNV sfRNA), 12.5 ng of pMT-Fluc-3'UTR, 3 ng of pMT-Rluc, and 2.5 ng of pMT-miRNA (either pri-dme-miRNA1 or pri-dme-miRNA12). Expression of the inducible constructs was induced 48 hpt by 5  $\mu$ M CuSO<sub>4</sub> and assayed 24 h postinduction.

*Aedes albopictus* U4.4 and *Aedes pseudoscutellaris* Ap61 mosquito cells were grown in Leibovitz L-15 medium (Invitrogen) supplemented with 10% heatinactivated FCS (Gibco), 2% tryptose phosphate broth (Sigma), and 1% nonessential amino acids (Invitrogen) at 28°C. Then, 1.5  $\times$  10<sup>5</sup> cells were seeded per well in a 24-well plate. Transfections were performed 24 h after seeding using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For the double-stranded RNA (dsRNA)-induced silencing assay, 8 ng of pAcIE1-*Renilla*, 50 ng of pIZ-Firefly, and 500 ng of the different pIB-vectors (MBP, MBP-HDVr, 3'UTR WNV, or 3'UTR DENV) were cotransfected in U4.4 cells. Silencing was induced after 24 h by transfection of 1.8 ng of dsRNA, using either Firefly luciferase-specific (dsFluc) or off-target (ds-scrambled) constructs. The cells were lysed 24 h after dsRNA transfection, and the luciferase expression was determined using a dual luciferase reporter assay (15).

The siRNA-induced silencing assay was performed as previously described (57). Briefly, Vero cells were seeded and transfected 24 h later with constructs expressing the sfRNA constructs, tombusvirus P19, or MBP-HDVr as a negative control. At 24 hpt, and the cells were cotransfected with specific (siFluc) or nonspecific siRNA (si-scrambled) molecules and constructs expressing Firefly luciferase and *Renilla* luciferase. The cells were lysed, and the luciferase expression was determined 48 h after the second transfection.

**SFV replicons.** SFV replicons were constructed as follows. The WNV 3'UTR-HDVr was PCR amplified from the existing insect expression plasmid (pIB-3'UTR WNV-HDVr) to introduce BamHI and XmaI sites for cloning behind the subgenomic 26S promoter of SFV-3H-Rluc replicon, resulting in SFV-3H-Rluc-3'UTR WNV. The SFV1-3H-Rluc replicon was generated by replacement of the BglII-SpeI restriction fragment from pSFV4-3H-Rluc (28) with the corresponding fragment from pSFV1. SFV-3H-Rluc-ECMV IRES was constructed in a similar way using BamHI and XmaI sites. SFV-3H-Rluc replicons were linearized by SpeI and *in vitro* transcribed by using a SP6 MEGAscript kit (Ambion) in the presence of cap analogue according to the manufacturer's protocol. *In vitro*-transcribed RNA was transfected in U4.4 cells 24 h after seeding in a 24-well plate (1.5  $\times$  10<sup>5</sup> cells/well). Luciferase expression was measured at 24 hpt using a *Renilla* luciferase assay.

**Statistics.** The relative luciferase expression (RL) was calculated as  $RL_i = I_{\text{Fluc},i} / I_{\text{Rluc},i}$ , where  $I$  is the measured intensity and  $i$  is the sample. To cancel out construct-specific effects, values under treatment (for example, cotransfection with shFluc) were normalized against the same construct that was treated with a negative control (in this example, sh-scrambled). Thus,  $NRL_x = RL_{i,\text{treated}} / RL_{i,\text{negative control}}$ .

Experiments were performed in duplicate or in triplicate and repeated independently at least twice. The independent experiments were averaged as follows:

$$\overline{NRL} = \sum_x \frac{NRL_x}{n}$$

where  $x$  is the  $x$ th experiment and  $n$  is the total number of experiments. The statistics were calculated using custom written scripts in R ([www.r-project.org](http://www.r-project.org)). In the case of pairwise testing a two-sample independent  $t$  test was performed, as provided by R. Multiple testing was done by applying Tukey's honestly significant difference test (Tukey's HSD; also known

as Tukey's range test), and the  $q$ -value was calculated and compared to the indexed  $q$ -value in the studentized range distribution available in R. Significant differences ( $P < 0.05$ ) are indicated in the figures by asterisks.

**dsRNA production and electromobility shift assays (EMSAs).** Radioactively labeled 114-nt dsRNA, siRNA, and miRNA molecules were produced as previously described (58). Briefly, the 114-nt dsRNA was generated by T7 RNA polymerase (Promega) transcription from an eGFP PCR product in the presence of [ $\alpha$ -<sup>32</sup>P]CTP (Perkin-Elmer), followed by incubation at 70°C for 10 min and cooling down slowly. After treatment with DNase I and RNase A, dsRNA molecules were loaded onto an 8% PAGE-0.5 $\times$  Tris-borate-EDTA (TBE) native gel and gel purified. siRNA and miRNA molecules were produced by end labeling of the GFP siRNA guide strand or the ath-miRNA171a strand using [ $\gamma$ -<sup>32</sup>P]ATP (Perkin-Elmer) and T4 polynucleotide kinase, followed by gel purification (22). The binding reaction was performed as previously described (24, 58). A total of 0.5 nM radiolabeled RNA was incubated for 20 min at room temperature with  $\sim$ 2  $\mu$ g of total protein from the cell extract. Complexes were separated on a native 0.5 $\times$  TBE acrylamide gel, either a 5% gel in the case of 114-nt dsRNA or an 8% gel for small dsRNAs, and then visualized by exposure to a phosphorimager screen overnight.

**Ago1/2 depletion in insect cells.** Templates for dsRNA production of *Drosophila melanogaster* Ago1 and Ago2 were produced by PCR using primers with T7 promoter sequences (Ago1 FW, TAATACGACTCACTA TAGGGAGAGTTCCTG TTACCTGAAGATCACC; Ago1 RV, TAATACG ACTCACTATAGGGAGAAGAAGAGTCGAGTGTGATGGC; Ago2 FW, TAATACGACTCACTATAGGGAGATACTATGGTGAAGAACGGG TCG; and Ago2 RV, TAATACGACTCACTATAGGGAGAGAACATGTC CTCAATCTCTCTCC). PCR products were gel purified and used for dsRNA production with the RNAi MEGAscript kit (Ambion). Experiments investigating the effect of Ago1 or Ago2 knockdown on the miR1 sensor constructs in S2 cells were basically performed as previously described (58). S2 cells were mixed with 200 ng of dsRNA of either Ago1, Ago2, or eGFP (unspecific) during seeding. Transfections were performed using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Knockdown efficiencies were checked by reverse transcription-PCR (RT-PCR) using the above primers and Western blot analysis using antibodies against Ago1/2 (Abcam, ab5070 and ab5072).

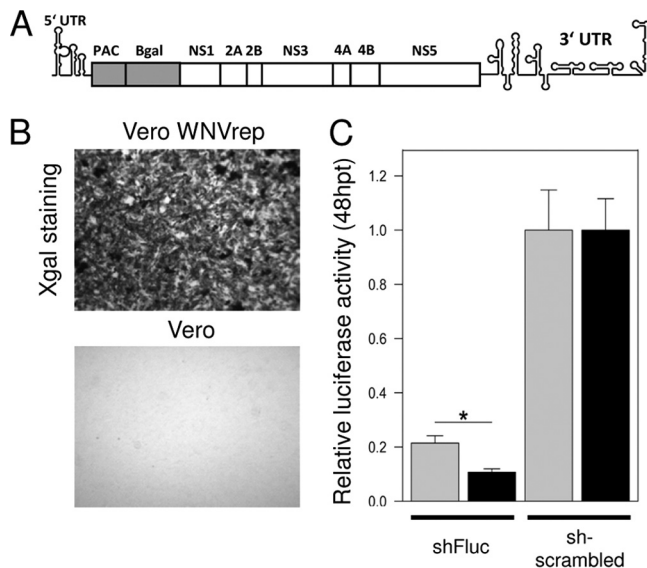
**In vitro Dicer assay.** dsRNA of 700 bp of the GFP gene was transcribed *in vitro* as described previously (58) using T7 RNA polymerase (Invitrogen). dsRNA was digested into siRNA by recombinant human Dicer (Genlantis), either in the absence or in the presence of decreasing amounts of *in vitro*-transcribed WNV sfRNA (52). Reaction products were loaded on an RNase-free agarose gel and stained with ethidium bromide.

**Immunofluorescence and Beta-galactosidase staining.** WNVrep cells expressing  $\beta$ -galactosidase were visualized using X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) staining. After being washed with phosphate-buffered saline (PBS), the cells were fixed for 10 min with 4% paraformaldehyde, followed by washing with PBS and the addition of X-Gal solution (53). The cells were incubated at 37°C, and blue cells were visualized by microscopy. Maintenance of viral replication in DENVrep cells was determined by immunofluorescence using a J2 monoclonal antibody that specifically recognizes long dsRNA and a rhodamine anti-mouse secondary antibody (Molecular Probes). Detection was performed as previously described (18).

## RESULTS

**WNV RNA replication impairs RNA silencing in mammalian cells.** To investigate whether West Nile virus (WNV) RNA replication had any effect on the efficiency of RNAi in mammalian cells, shRNA-induced silencing assays were performed in either wild-type Vero cells or Vero cells stably expressing a WNV replicon. Stable WNV replicon cells were established by transfecting Vero cells with *in vitro*-transcribed WNV replicon RNA expressing  $\beta$ -galactosidase as the reporter protein (Fig. 1A), followed by





**FIG 1** WNV RNA replication suppresses shRNA-mediated gene silencing in mammalian cells. (A) Schematic representation of the puromycin-selectable WNV replicon, encoding  $\beta$ -galactosidase (Bgal) as a reporter. UTR, untranslated region; PAC, puromycin acetyltransferase; NS, nonstructural protein. (B) WNV replication in Vero cells expressing the WNV replicon (WNVrep) was verified by  $\beta$ -galactosidase detection using X-Gal staining (upper panel). Wild-type Vero cells were stained as a control (lower panel). (C) Suppression of shRNA-induced silencing in Vero WNVrep cells (gray) or normal Vero cells (black). Cells were cotransfected with Firefly luciferase (Fluc), *Renilla* luciferase (Rluc), and shRNA, either Fluc-specific (shFluc) or off-target (sh-scrambled). Normalized relative luciferase expression (Fluc/Rluc) was determined at 48 hpt, and the means of four independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance determined by an independent two-sample Student *t* test ( $P < 0.05$ ).

puromycin selection (36, 53). X-Gal staining of puromycin-resistant WNV replicon cells, with nontransfected Vero cells as the negative control, confirmed a successful selection procedure (Fig. 1B). Vero cells with or without WNV replicon were transfected with plasmids encoding Firefly luciferase as the reporter, *Renilla* luciferase as internal transfection control, and a plasmid expressing either a shRNA against firefly luciferase (shFluc) or off-target (sh-scrambled) (57). The sequence-specific silencing of Fluc by shFluc was significantly less efficient in WNV replicon cells compared to silencing in normal Vero cells, with 22% versus 10% relative luciferase activity, respectively (Fig. 1C). These results show that WNV RNA replication has a negative effect on the efficiency of shRNA-induced RNA silencing in mammalian cells.

**WNV nonstructural proteins do not suppress RNAi in mammalian cells or in plants.** Interference with the (antiviral) RNA silencing machinery is a common feature of plant and insect viruses, which encode specialized proteins called RNA silencing suppressors (RSSs). Even in some mammalian viruses, proteins have been reported with apparent RSS activity, e.g., NS1 of influenza A virus or Ebola virus VP35 (9, 21, 63). The majority of these RSSs act by binding to dsRNA, which are key molecules in the mammalian IFN response but also in the RNA silencing pathways (13).

In order to find an explanation for the observed reduced efficiency of RNAi in flavivirus replicon cells (Fig. 1B), we examined whether any of the WNV products, i.e., nonstructural proteins

and/or sfRNA, could act in a fashion similar to other RSS proteins by binding dsRNA molecules.

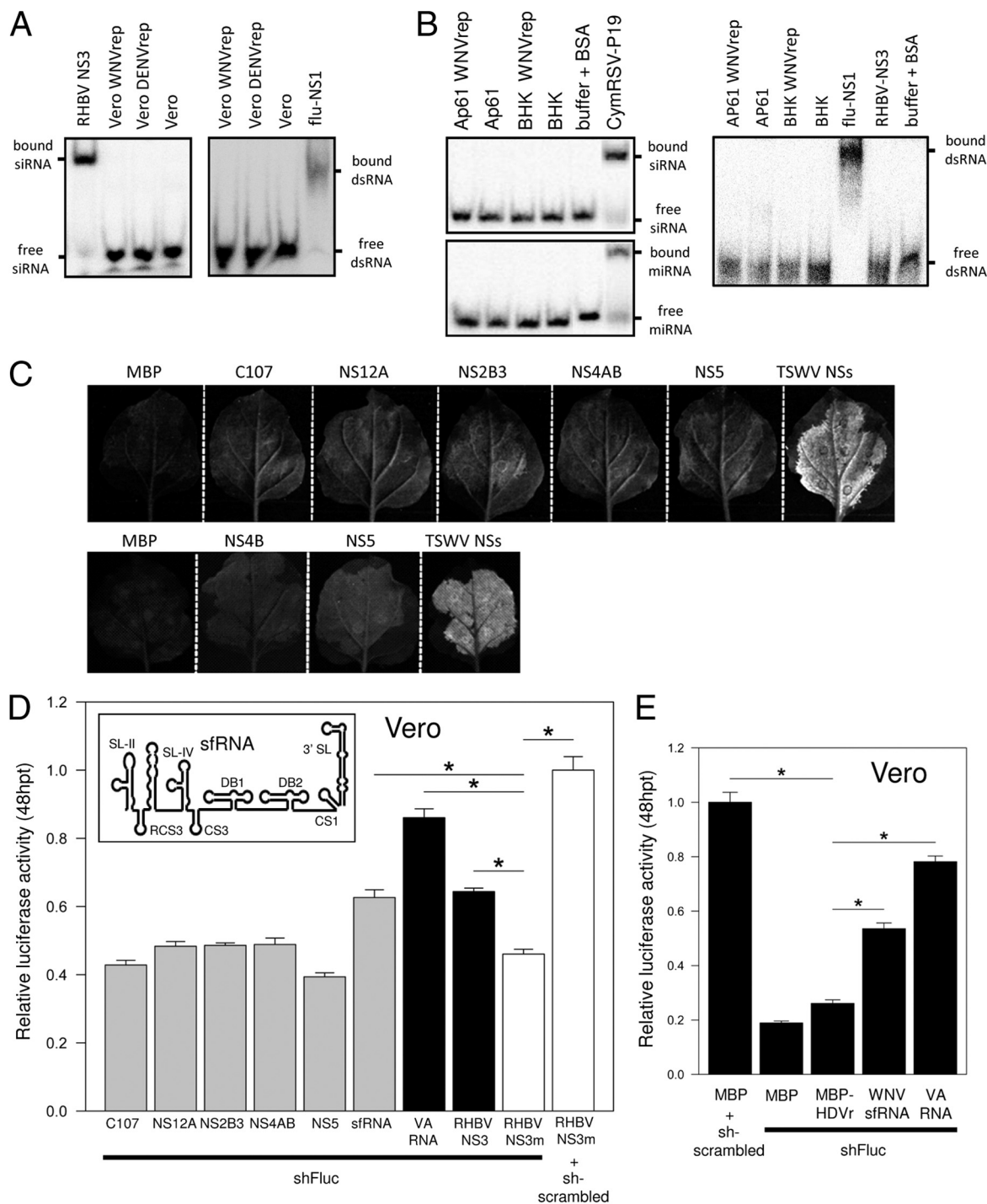
First, in an EMSA, cell extracts of Vero or Vero-WNVrep cells were mixed with either radiolabeled siRNA or long 114-nt dsRNA molecules and loaded onto native acrylamide gels (Fig. 2). No retardation could be observed for any of the investigated extracts, in contrast to the positive controls, RHBV-NS3 (binding exclusively siRNA [24]) (Fig. 2A, left) and influenza virus NS1 (binding long dsRNA [70]) (Fig. 2A, right). To rule out putative cell line-specific differences, WNV replicon cell lines were also established in mosquito (Ap61) and rodent (BHK-21) cells, and cell extracts were tested in a similar EMSA (Fig. 2B). Again, no retardation of si/miRNA (Fig. 2B, left) or long dsRNA (Fig. 2B, right) could be observed for any of the investigated extracts. Together, these results suggest that none of the nonstructural proteins of WNV was able to efficiently bind small or longer dsRNA molecules in this experimental setup.

Other studies suggested that none of the mature DENV proteins showed activity as RSSs (32). To investigate whether similar results could be obtained with WNV proteins, we cloned them and tested for RSS activity in reporter-based plant (Fig. 2C) and mammalian (Fig. 2D) RNAi suppressor assays (9, 57). The WNV capsid protein was also cloned and tested, since only a truncated version (20 amino acids) of capsid is expressed from the WNV replicon in Fig. 1 (27).

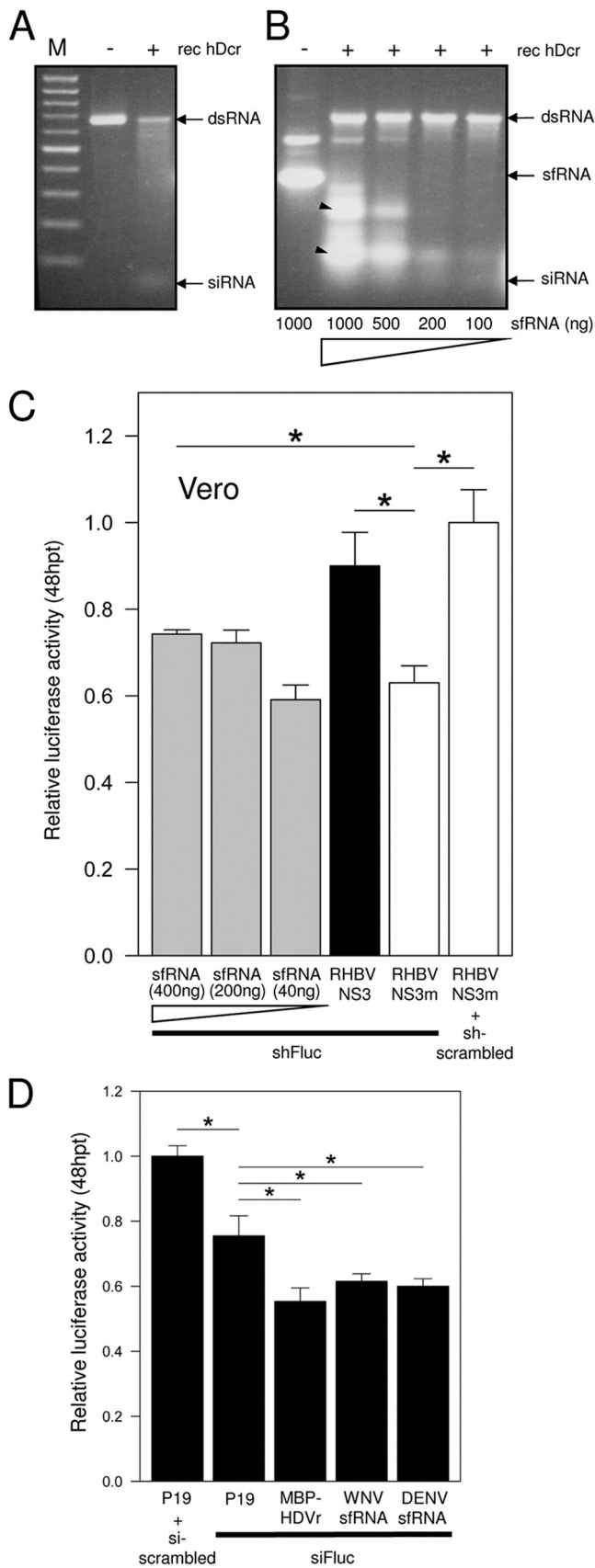
Clearly, none of the WNV nonstructural (NS) proteins nor the capsid (C) protein could suppress reporter gene silencing in plants (Fig. 2C) or mammalian cells (Fig. 2D), in contrast to the positive controls, TSWV-NSs and RHBV-NS3, which are potent RSSs used in plant and mammalian cells, respectively (57, 58). In both assays, the expression of WNV NS1, NS3, and NS5 was confirmed by Western blot analysis (data not shown). There is a possibility that the expression levels of WNV products were not high enough to display RSS activity; however, the positive controls in the plant and mammalian RNAi suppressor assays (TSWV-NSs and RHBV-NS3, respectively) were produced from the same expression vectors and worked efficiently (Fig. 2C and D). Overall, these results suggest that the observed interference of WNV replicon RNA replication with shRNA-induced RNAi is independent of WNV-encoded viral proteins.

**Noncoding flavivirus RNA of WNV suppresses shRNA-induced silencing in mammalian cells.** Since WNV proteins did not suppress RNAi, the question remained which WNV products produced from replicon RNA are able to suppress RNAi? Therefore, we also tested in our assays a plasmid producing a noncoding WNV RNA product called sfRNA (52). sfRNA is an abundantly expressed, 3' UTR-derived RNA molecule 525 nt in length, with a complex RNA structure containing many stem-loops (19, 52), and shares some structural similarity to the RSS of adenovirus VA RNAI/II (3). A plasmid expressing VA RNA was included in the mammalian RNAi suppressor assay as an additional positive control.

In contrast to the result obtained for the WNV nonstructural proteins, adenovirus VA RNA very efficiently suppressed shRNA-induced silencing in mammalian cells, with 87% relative luciferase activity (Fig. 2D). Notably, sfRNA of WNV was also able to suppress RNAi, with an efficacy (63% relative luciferase activity) similar to that of the positive control RHBV-NS3 (64% relative luciferase activity) but slightly less efficient than VA RNA (Fig. 2D). The remote possibility that the short hepatitis delta virus



**FIG 2** WNV sRNA inhibits shRNA-induced silencing in mammalian cells. (A and B) Electromobility gel shift analysis performed by incubating cell lysates of normal Vero, BHK, Ap61, or Vero WNVrep/DENVrep, BHK WNVrep, Ap61 WNVrep cells with radiolabeled siRNA molecules, ath-miRNA171/miRNA171\* duplex miRNA molecules, or 114-nt radiolabeled dsRNA molecules for 20 min at room temperature. RNA-protein complexes were separated on a native polyacrylamide gel, dried and exposed overnight to a phosphorimager screen. MBP-NS3 of RHBV, P19 of CymRSV, or influenza virus NS1 were used as positive controls. (C) WNV NS1-5 does not suppress RNA silencing in plants. *Agrobacterium tumefaciens* harboring vectors encoding mGFP were coinfiltrated in *Nicotiana benthamiana* leaves with MBP (negative control), *Tomato spotted wilt virus* (TSWV) NSs (positive control), or different WNV proteins (C107 [capsid], NS12A, NS2B3, NS4AB, and NS5) constructs, respectively. Green fluorescence under UV light was determined in infiltrated leaves 5 days after agroinfiltration. (D) Suppressor activity of WNV proteins and sRNA on shRNA-induced silencing in Vero cells. Luciferase activity of cells cotransfected with Firefly luciferase (Fluc), *Renilla* luciferase (Rluc), a specific (shFluc) or off-target (sh-scrambled) shRNA, and different WNV proteins (C107 [capsid], NS12A, NS2B3, NS4AB, and NS5) or WNV sRNA (gray bars) was measured at 48 hpt. The NS3 of RHBV and VA RNA were used as positive controls (black bars) and a NS3 mutant (NS3m RHBV) as negative control (white bar). The means of two independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance by Tukey's HSD ( $P < 0.05$ ). (Inset) Schematic representation of the sRNA structure. SL, stem-loop; DB, dumbbell; RCS, repeated conserved sequence; CS, conserved sequence. (E) To determine the lack of putative RNAi suppressor activity by the added HDVr sequence, the experiments performed for panel D were repeated with MBP, MBP-HDVr, WNV-sfRNA, and VA RNA. The means of two independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance determined by Tukey's HSD ( $P < 0.05$ ).

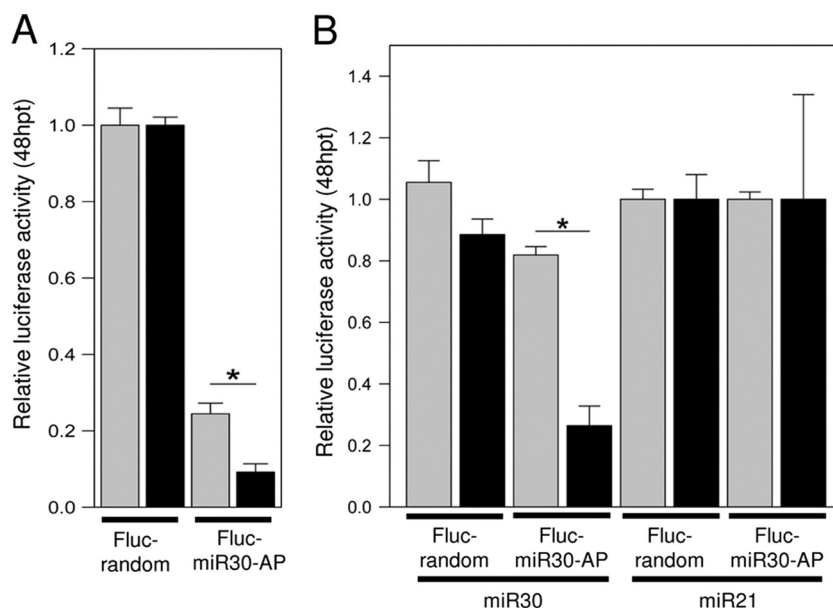


ribozyme (HDVr) sequence present in WNV replicons and the WNV sfRNA constructs could suppress RNA silencing was ruled out by including a plasmid expressing MBP-HDVr as a control, lacking any RSS activity (Fig. 2E). Taken together, the results show that WNV sfRNA is able to suppress shRNA-induced RNAi in mammalian cells.

**WNV sfRNA is processed by human Dicer *in vitro* and suppresses RNAi in a concentration-dependent manner.** sfRNA is abundantly expressed during infections of all flaviviruses in cultured cells of both vertebrate and invertebrate origin (52). WNV sfRNA represents the last 525 nt of the 3' UTR and is predicted to contain several stem-loop structures (40, 52) with structural similarity to VA RNAI/II and even to pre-miRNA structures, both of which are known to be processed by Dicer (4). The highly structured adenovirus VA RNAI/II molecules, expressed at very high concentrations during adenovirus infection, bind to RNAi proteins and thereby oversaturate and block the natural silencing machinery (3).

To further elucidate the mechanism of sfRNA suppression of RNAi, we investigated whether sfRNA could interfere *in vitro* with processing of long dsRNA templates by Dicer. A Dicer cleavage assay with *in vitro*-transcribed WNV sfRNA, long dsRNA (Dicer substrate) and recombinant human Dicer was performed (Fig. 3). As expected, Dicer was able to digest the long dsRNA template, leading to the production of siRNA (Fig. 3A, bottom arrow). However, when sfRNA was added to the reaction mixture in increasing concentrations, the *in vitro* processing of dsRNA into siRNA was severely inhibited (Fig. 3B). It should be noted that in addition to the inhibition of Dicer processing of long dsRNA, sfRNA itself was also processed into smaller cleavage products (Fig. 3B, arrowheads), suggesting that sfRNA acted as a competing Dicer substrate in this assay. We were unable to determine the specific cleavage sites within the sfRNA, but it could be hypothesized that cleavage would most likely occur within the double-stranded stem-loop structures that are abundantly present in sfRNA. Whereas these results were obtained in a rather artificial experimental *in vitro* setting with a limited number of biological components (Dicer and RNA), a similar concentration-dependent inhibition of WNV sfRNA on RNAi was also observed in a

**FIG 3** sfRNA interferes with Dicer cleavage of dsRNA *in vitro* and suppresses RNAi in a concentration-dependent manner. (A and B) dsRNA of 700 bp was incubated either in the absence (A) or in the presence (B) of decreasing amounts of *in vitro*-transcribed WNV sfRNA with human recombinant Dicer and loaded onto an ethidium bromide-stained agarose gel. (C) *Renilla* luciferase (Rluc), Firefly luciferase (Fluc), either specific (shFluc) or off-target (sh-scrambled) shRNA, and decreasing concentrations of WNV sfRNA were cotransfected into Vero cells. RHBV NS3 or a dysfunctional mutant (RHBV NS3m) were used as positive and negative controls, respectively. Relative luciferase expression (Fluc/Rluc) was determined at 48 hpt and normalized to the cells transfected with off-target shRNA. The means of two independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance determined by Tukey's HSD ( $P < 0.05$ ). (D) Bypassing of sfRNA-mediated RNAi suppression by siRNA transfection. *Renilla* luciferase (Rluc), Firefly luciferase (Fluc), either specific (siFluc) or off-target (si-scrambled) siRNA, and plasmids expressing WNV/DENV sfRNA were cotransfected into Vero cells. Tombusvirus P19, which binds siRNA, was used as a positive control. The relative luciferase expression (Fluc/Rluc) was determined at 48 hpt siRNA transfection and normalized to the cells transfected with off-target siRNA. The means of four independent experiments performed in triplicate are shown with the standard errors. Asterisks indicate significance determined by Tukey's HSD ( $P < 0.05$ ).



**FIG 4** Suppression of the mammalian miRNA pathway in WNV replicon cells. (A) Vero cells either expressing the WNV replicon (Vero-WNV [▨]) or lacking the replicon (Vero wild type [■]) were transfected with expression vectors encoding pCMV-luc-miR30-AP or pCMV-luc-random alone or in combination with either a specific miRNA (hsa-miR30) or off-target (hsa-miR21). The results shown are the means of two independent experiments performed in duplicate. Asterisks indicate significance determined by independent two-sample Student *t* tests ( $P < 0.05$ ). (B) The luciferase expression was measured 2 days posttransfection, and the relative luciferase expression (Fluc/Fluc) was determined. The luciferase level measured with nonspecific hsa-miR21 was set at 1.0. The results shown are representative of three independent experiments performed in duplicate. Asterisks indicate significance determined by an independent two-sample Student *t* test ( $P < 0.05$ ).

shRNA-induced silencing suppressor assay in mammalian cells (Fig. 3C).

Interference with Dicer processing suggests that sfRNA acts as RSS upstream of RISC. In this scenario, transfected siRNA should be capable of bypassing RSS activity, since it is directly loaded into RISC independent of Dicer. We therefore examined whether or not sfRNA could suppress siRNA-mediated RNAi. Vero cells were transfected with luciferase reporter plasmids and with a sfRNA-expressing plasmid. Next, the cells were transfected with Fluc-specific siRNA. Fluc activity was measured 48 h after siRNA transfection. The results showed that P19, a strong RSS with high affinity to siRNA, but not the sfRNAs of WNV and DENV could suppress siRNA-induced RNAi (Fig. 3D).

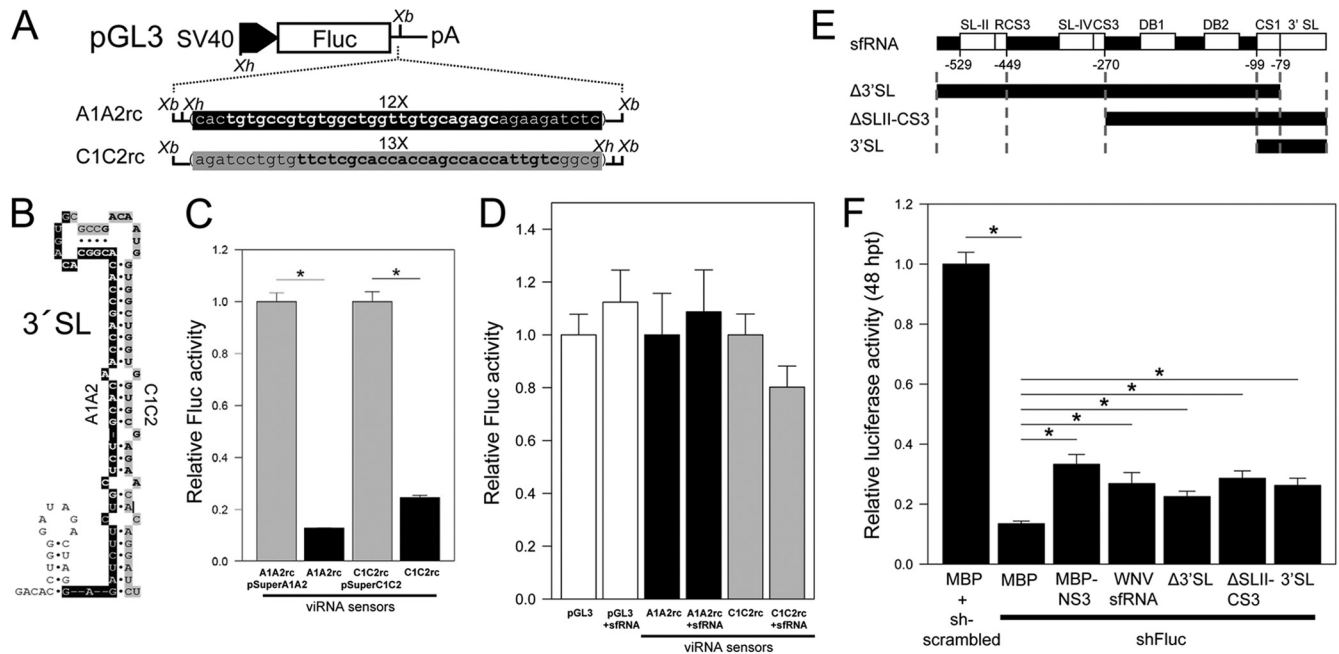
Taken together, the results suggest that WNV sfRNA, as was previously reported for VA RNAI/II (3), can interfere in a concentration-dependent manner with Dicer processing of dsRNA *in vitro* and of shRNA-induced RNAi pathway *in vivo*. Bypassing of this interference with transfected siRNA suggests that the inhibitory effect of sfRNA on RNAi activity is upstream of RISC.

**WNV RNA replication interferes with the endogenous miRNA pathway in mammalian cells.** The siRNA pathway in insects and plants has clearly been demonstrated to be involved in antiviral responses, but the role of the siRNA pathway in an antiviral response in mammals is the subject of debate (12). At the same time, the presence and importance of the miRNA pathway in mammalian gene regulation is well accepted (2). In contrast to plants and insects, mammals code for only one Dicer enzyme, and no clear distinction exists between siRNA and miRNA pathways. Interference of an RSS with mammalian Dicer is therefore expected to negatively affect RNAi induced by both siRNA and miRNA molecules.

To investigate whether sfRNA indeed would also interfere with the endogenous miRNA pathway in mammalian cells, a previously described Firefly luciferase-based miRNA reporter assay was performed using a Fluc sensor construct harboring multiple human miRNA-30 (hsa-miR30) target sites in the 3' UTR of the Fluc mRNA (76). This sensor construct, Fluc-miR30-AP, is silenced by endogenously produced miR30 in Vero cells. As expected, cotransfection of the Fluc-miR30-AP sensor construct and *Renilla* luciferase as an internal transfection control resulted in decreased Fluc expression (10% relative luciferase activity) compared to cells transfected in a similar way with a sensor construct harboring randomized miRNA target sites (Fig. 4A). However, in Vero-WNVrep cells, a significant suppression of miR30-induced silencing (26% in comparison to 10% relative luciferase activity in normal Vero cells) was observed (Fig. 4A). To demonstrate that the observed gene silencing via miR30 was truly sequence specific, the experiment was repeated in a background of overexpression of the homologous hsa-miR30 and a heterologous hsa-miR21 (miR21). Again, cotransfection of the Fluc-miR30-AP sensor construct in Vero cells, together with a plasmid overexpressing hsa-miR30 (76), led to silencing (28% relative luciferase activity) of the Fluc signal. The observed silencing was sequence specific, because in cells transfected with a plasmid expressing hsa-miR21 (76) no silencing was observed (Fig. 4B). In Vero-WNVrep cells, a significant suppression of miR30-induced silencing (82% compared to 28% relative luciferase activity in normal Vero cells) was observed (Fig. 4B). In conjunction with the experiments shown earlier, the results presented here demonstrate that WNV RNA replication suppresses both siRNA- and miRNA-induced RNAi in mammalian cells.

**RSS activity of sfRNA is not restricted to the 3' SL.** In mosquito cells, we have recently shown that the 3' SL (Fig. 2D, inset) in





**FIG 5** Mapping of RNAi suppressor activity within WNV sRNA. (A to D) 3' SL-derived miRNA sensor assay using Firefly luciferase-based sensor constructs. (A) Schematic representation of miRNA sensor constructs for expression in mammalian cells. Reverse complement (rc) of A1A2 and C1C2 tandem repeats are indicated in black and gray, respectively. Sequences used for shRNA cloning into pSuper plasmids are indicated in boldface. Fluc, Firefly luciferase; SV40, simian virus 40 promoter; pA, polyadenylation signal; Xb, XbaI restriction site; Xh, XhoI restriction site. (B) Schematic representation of the WNV 3' SL. A1A2 and C1C2 sequences for tandem repeat cloning into miRNA sensor constructs are indicated in black and gray, respectively. (C) Functionality of miRNA sensor constructs. Cells were cotransfected with pRL-TK, pGL3-(sensor constructs), and either pSuper-A1A2 or pSuper-C1C2 or control plasmid. The relative luciferase expression (Firefly/*Renilla*) was determined at 24 hpt. The means of two independent experiments performed in triplicate are shown with the standard errors. Significance was tested by an independent two-sample Student *t* test ( $P < 0.05$ ). (D) Silencing of miRNA sensor constructs by sRNA expression in Vero cells. Cells were cotransfected with pRL-TK, pGL3-(sensor constructs), and either pDEST40-sRNA or control plasmid. The relative luciferase expression (Firefly/*Renilla*) was determined at 24 hpt. The means of three independent experiments performed in duplicate are shown with the standard errors. Significance was tested by an independent two-sample Student *t* test ( $P < 0.05$ ). (E) Schematic representation of WNV sRNA with abbreviations as in Fig. 2. The numbers are nucleotide positions from the 3' terminus of the WNV 3' UTR. Deletions within sRNA are indicated. (F) Suppressor activity of WNV sRNA truncations on shRNA-induced silencing in Vero cells. The luciferase activity of cells cotransfected with Firefly luciferase (Fluc), *Renilla* luciferase (Rluc), a specific (shFluc) or off-target (sh-scrambled) shRNA, and WNV sRNA variants was measured at 48 hpt. The NS3 of RHBV was used as a positive control, and MBP was used as a negative control. The means of two independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance determined by Tukey's HSD ( $P < 0.05$ ).

the 3'UTR (and thus sRNA) of WNV is a precursor for a viral miRNA (25) capable of silencing a miRNA reporter construct. To obtain further insight into the mechanism of sRNA-mediated RNAi suppression, we asked the question whether putative miRNA production from the sRNA 3' SL was correlated with the observed Dicer inhibition and RSS activity in both mammalian and insect cells. The miRNA reporter plasmids contain a CMV promoter for expression in mammalian cells (Fig. 5A) and encode a Fluc gene with a 3'UTR containing 3' SL-derived miRNA target sequences called A1A2rc and C1C2rc, which are reverse complemented to the sequences in the 3' SL (Fig. 5B). First, to verify the functionality of the pGL3-based miRNA sensor constructs, they were cotransfected with pSuper plasmids expressing siRNA specifically targeting either side of the 3' SL (A1A2 or C1C2) (Fig. 5C). This experiment indicated that the A1A2 and C1C2 repeat regions cloned in both orientations in pGL3 in the 3'UTR of Fluc were efficiently targeted (>90% silencing of Fluc) by complementary siRNA generated from pSuper plasmids, indicating that the sensor constructs were functional. Next, sRNA was cotransfected along with the miRNA reporter plasmids (Fig. 5D). The result showed that neither the A1A2rc nor the C1C2rc reporter transcript was significantly silenced in the presence of sRNA. This results sug-

gests that sRNA is unlikely to be a source of 3' SL-derived miRNA in mammalian cells in contrast to insect cells (25).

Since no viral miRNA derived from the 3' SL could be identified in the miRNA sensor assay, we sought to determine whether other RNA structures within sRNA could contribute to the observed RSS activity. A number of deletions (from SL-II to CS3, from SL-II to CS1, and 3' SL only) (52) were introduced in sRNA (Fig. 5E), and plasmids expressing the truncated sRNA variants were tested in an RNAi suppressor assay as before (Fig. 5F). The results indicated that all sRNA variants still displayed RSS activity, including the relatively short variant consisting of only the CS1 and 3' SL RNA structures. This result suggested that more than a single RNA structure within sRNA was involved in RSS activity.

**sRNA suppresses short hairpin RNA-induced silencing in insect cells.** Like many other arboviruses, WNV is transmitted by mosquitoes, actively replicates in mosquito tissues, and eventually accumulates in the salivary glands prior to virus transmission to the vertebrate host via blood-feeding. A characteristic of most arbovirus infections is the limited pathology in the infected insect and the establishment of persistent virus replication (17). RNAi is implicated to be a determining factor for successful arbovirus in-

fection of the mosquito (6). It has been shown that heterologous RSS, e.g., FHV-B2, can enhance alphavirus replication in mosquitoes (11). To determine whether sfRNA could have a similar effect on alphavirus replication, we engineered sfRNA into a Semliki Forest virus (SFV) replicon and determined the level of replication in RNAi-competent U4.4 cells. As a control, we included a SFV replicon expressing another, unrelated, highly structured RNA derived from the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (Fig. 6A). Both replicons expressed *Renilla* luciferase (Rluc) from the nonstructural polyprotein as described previously (28). The results show that SFV replication in mosquito cells is enhanced upon coexpression in *cis* of sfRNA (Fig. 6B).

To determine the effect of WNV sfRNA on the siRNA pathway in insect cells, a dsRNA-mediated silencing assay using luciferase reporters was developed for mosquito cells to allow easy quantification. To this end, *Aedes albopictus* U4.4 cells were cotransfected with plasmids encoding Fluc, Rluc (internal transfection control) and either MBP or WNV sfRNA. At 24 hpt, silencing was induced by transfection of dsRNA, either specifically against Firefly luciferase (dsFluc) or off-target (ds-scrambled), and the luciferase activity was determined at 48 hpt. Cells cotransfected with MBP, Fluc, and dsFluc carrying plasmids showed silencing of Fluc expression (ca. 47% relative luciferase activity), which was not observed with ds-scrambled (Fig. 6C). In the case of cotransfection of WNV-sfRNA with Fluc and dsFluc, no significant reduction of Fluc activity could be detected, indicating that sfRNA very efficiently suppressed dsRNA-induced silencing (Fig. 6C).

To ensure that the observed effect of sfRNA was not an anomaly of dsRNA-induced silencing in mosquito U4.4 cells, a similar experiment was performed in *Drosophila melanogaster* S2 cells, using a shRNA-mediated silencing assay. In this experiment, S2 cells were cotransfected with inducible plasmids encoding Fluc, Rluc (internal control), and shRNA constructs specifically targeting Fluc (shFluc) or a control shRNA targeting GFP (shGFP). Next, Fluc, Rluc, and shRNA expression was induced by the addition of CuSO<sub>4</sub> to the medium at 24 hpt, and the Fluc activity was measured at 48 hpt and normalized to the Rluc readings (Fig. 6D). Cells cotransfected with MBP, Fluc, and shFluc plasmids showed silencing of Fluc expression (ca. 55% relative luciferase activity), which was not observed with the shGFP-negative control (Fig. 6D). When a plasmid expressing WNV sfRNA was cotransfected with Fluc and shFluc, however, Fluc silencing was suppressed with an efficiency similar to that of the positive control RHBV-NS3 (Fig. 6D) (24), with 81 and 83% relative luciferase activities, respectively. Similar to what was observed in mammalian cells, the RSS activity of WNV sfRNA was concentration dependent (Fig. 6D). In conclusion, these experiments show that WNV sfRNA is able to interfere with the siRNA-based pathway in insect cells.

**sfRNA suppresses the induced miRNA pathway in insect cells.** In addition to the antiviral siRNA pathway, insects also have a functional miRNA pathway that has a function in gene regulation similar to that seen, for example, in mammals. In contrast to mammals, however, insects have two distinct Dicer enzymes, Dcr-1 and Dcr-2, instead of one, with dedicated functions in the miRNA or siRNA pathway, respectively (1). Although no clear antiviral activity has been attributed to the miRNA pathway in insects, the question remained as to whether WNV sfRNA, apart from its activity in inhibiting the siRNA pathway, could also in-

terfere with the miRNA pathway in insect cells. Several viral RSS proteins have been demonstrated to be able to interfere with both the siRNA and the miRNA pathways in different organisms (14, 58).

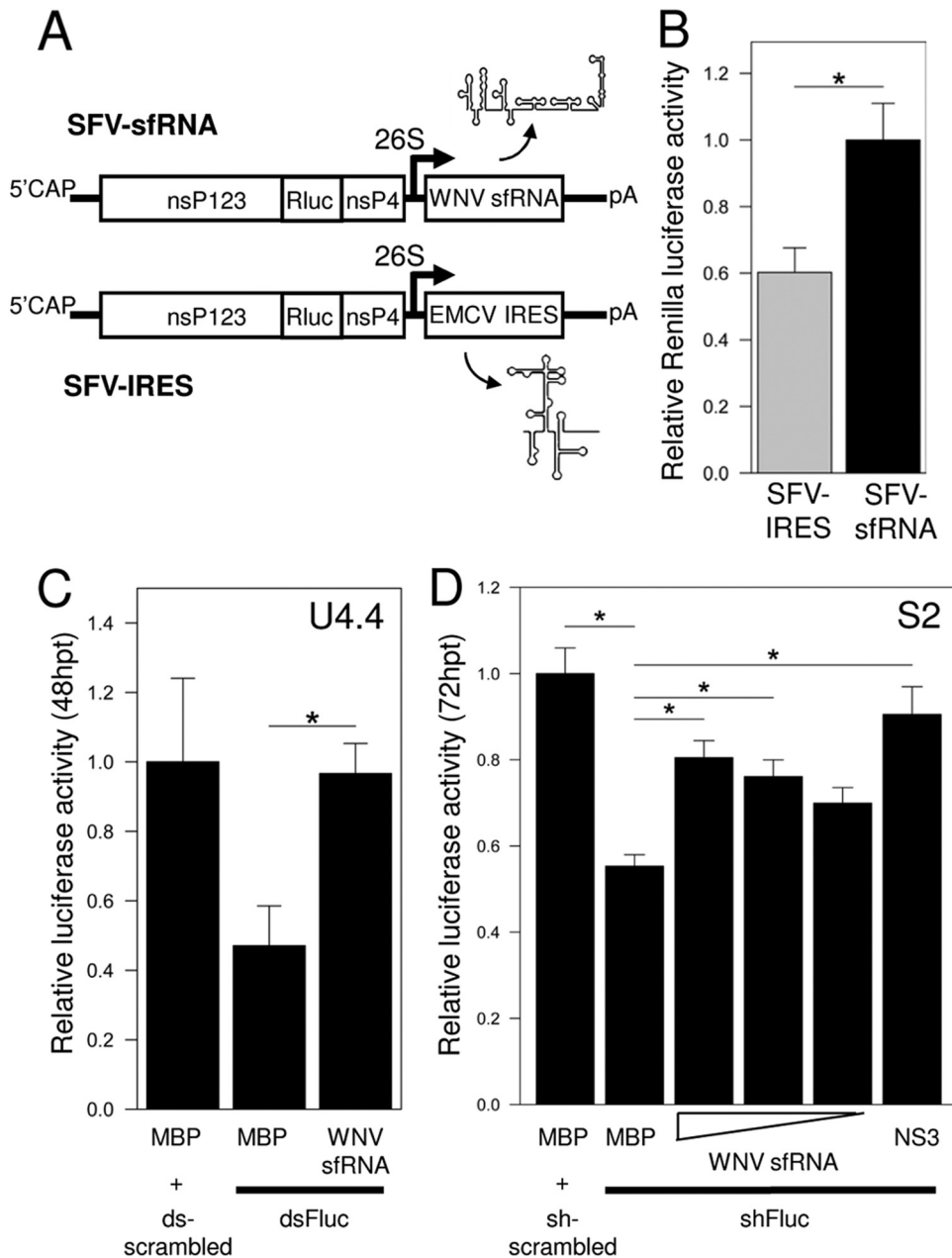
*D. melanogaster* S2 cells were cotransfected with an inducible Fluc dme-miRNA1-sensor construct (Fluc-3'UTR), vectors encoding either specific dme-miRNA1 or off-target dme-miRNA12, Rluc (internal transfection control), and either WNV sfRNA, MBP (negative control), or *Carnation Italian ringspot virus* (CIRV) P19 (positive control). As expected, a decrease in Fluc expression (ca. 48% relative luciferase activity) was observed in cells cotransfected with MBP, Fluc-3'UTR, and dme-miRNA1 but not with the off-target dme-miRNA12 (Fig. 7A). To make sure that the observed Fluc silencing was the result of miRNA1 and thus independent of the siRNA pathway, we checked that the miRNA1-induced silencing was lost in cells treated prior to transfection with dsRNA specifically targeting *Ago1* but not *Ago2* or the nonspecific GFP (Fig. 7B). Successful depletion of *Ago1* and *Ago2* transcripts and proteins was checked by RT-PCR and Western blotting, respectively (Fig. 7C). Since *Ago1* and *Ago2* are known to be predominantly loaded with miRNA and siRNA, respectively, these results confirmed that the miRNA pathway in insect cells was capable of silencing the Fluc reporter construct harboring dme-miRNA1 target sites.

After the demonstration of sequence-specific silencing by miRNA1 through the miRNA pathway, the putative suppressor effect of WNV sfRNA was investigated. A significant and reproducible rescue of Fluc expression was observed in the presence of WNV sfRNA, to a level similar to that observed for CIRV P19 (Fig. 7A), suggesting that WNV sfRNA interferes with the miRNA pathway in insect cells. Collectively, these results demonstrate that WNV sfRNA interferes with both the siRNA- and the miRNA-based pathways in insect cells.

**DENV also displays RSS activity in mammalian and insect cells.** sfRNA is produced by all flaviviruses (52, 60). To determine whether the observed interference with the RNA silencing pathways is common among flaviviruses, the same experiments were conducted with DENV1.

First, Vero cells stably expressing a DENV1 replicon (Vero-DENVrep) were established by transfection of Vero cells with DENV1 replicon RNA harboring an IRESneo cassette (54). Stable replicon cells were selected with G418. Active DENV replicon RNA replication was confirmed by immunofluorescence using the J2 anti-dsRNA antibody (Fig. 8A). Vero-DENVrep cells showed less silencing (ca. 28% relative luciferase activity) if transfected with shRNA specific against Firefly luciferase in comparison to wild-type Vero cells (10% relative luciferase activity) (Fig. 8B).

DENV sfRNA was also tested in the shRNA-mediated silencing assays in mammalian (Vero) and RNAi-competent mosquito (U4.4) cells and displayed a similar RSS activity (Fig. 8C and D) compared to WNV sfRNA (Fig. 2D and E and Fig. 8C). Furthermore, no RNAi suppressor activity of DENV1 nonstructural proteins was detected in the plant suppressor assay (data not shown), and no retardation of either small or long dsRNA was observed in DENV replicon cell extracts (Fig. 2A). These results show that the ability of sfRNA to interfere with the RNA silencing is observed for both WNV and DENV, in mammalian as well as in insect cells.

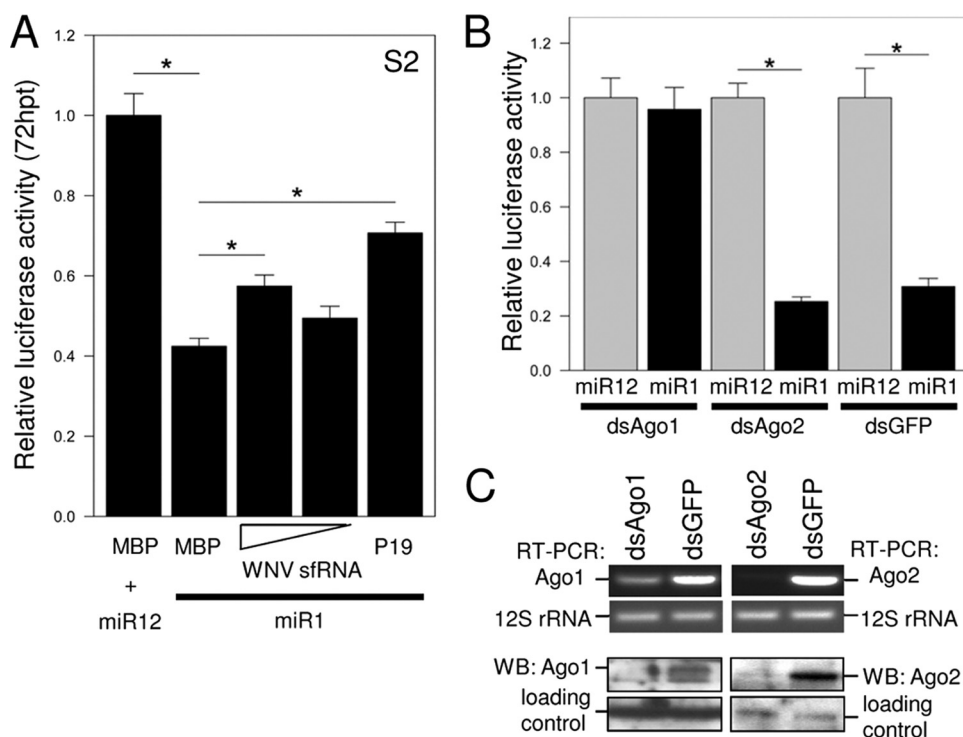


**FIG 6** WNV sfRNA interferes with siRNA-induced silencing in insect cells. (A) Schematic representation of SFV constructs. The *Renilla* luciferase (Rluc) gene is inserted upstream of nsP4. sfRNA or the EMCV IRES sequence is inserted downstream of the 26S subgenomic promoter. Schematic RNA structures of sfRNA and the EMCV IRES are shown. (B) Capped, *in vitro*-transcribed SFV RNA was transfected in *Aedes albopictus* U4.4 mosquito cells. Rluc expression was measured at 24 hpt. The means of four independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance determined by independent two-sample Student *t* test ( $P < 0.05$ ). (C) *Aedes albopictus* U4.4 mosquito cells were cotransfected with Firefly luciferase (Fluc), *Renilla* luciferase (Rluc), and MBP or WNV sfRNA constructs. After 24 h, silencing was induced by transfection of either specific (dsFluc) or off-target (ds-scrambled) *in vitro*-transcribed dsRNA. The luciferase activity was measured at 48 hpt, and the normalized relative luciferase activity (Fluc/Rluc) is shown with the standard errors (means of two independent experiments performed in duplicate). Asterisks indicate significance determined by Tukey's HSD ( $P < 0.05$ ). (D) Concentration-dependent effect of sfRNA on shRNA-induced silencing determined in *D. melanogaster* S2 cells by transfection of decreasing concentrations of WNV-sfRNA (140, 50, and 20 ng) construct in concert with Firefly luciferase (Fluc), *Renilla* luciferase (Rluc), and either specific (shFluc) or off-target (sh-scrambled) shRNA. RHBV NS3 was used as a positive control. The luciferase activity was measured at 48 hpt, and the normalized relative luciferase activity (Fluc/Rluc) is shown with the standard errors (means of three independent experiments performed in duplicate). Asterisks indicate significance determined by Tukey's HSD ( $P < 0.05$ ).

## DISCUSSION

RNA silencing is known as an important antiviral response in insects and plants. Until now, all investigated plant and “true” insect viruses have been shown to encode RSS proteins, which are

essential for the establishment of a successful viral infection. Our results and those of others (32) show that no proteins with RSS activity have thus far been identified in flaviviruses, despite the fact that these viruses are successfully infecting their mosquito vector.



**FIG 7** WNV sfRNA interferes with Ago1-dependent miRNA-induced silencing in insect cells. (A) Suppression of dme-miRNA1-induced silencing in S2 cells by cotransfection of a pMT-*Renilla* luciferase (Rluc), pMT-Firefly luciferase (Fluc)-dme-miRNA1 sensor construct, either specific (dme-miRNA1) or off-target (dme-miRNA12) primary miRNA and either MBP, CIRV P19 (as a positive control), or WNV sfRNA (180 or 50 ng). After induction at 48 hpt, the relative luciferase expression (Firefly/*Renilla*) was determined at 72 hpt, and the means of three independent experiments in duplicate are shown with the standard errors. Asterisks indicate significance by Tukey's HSD ( $P < 0.05$ ). (B) Silencing of the pMT-Firefly luciferase (Fluc)-dme-miRNA1 sensor construct is Ago1 dependent. S2 cells were soaked with 200 ng of dsRNA either Ago1, Ago2, or EGFP (unspecific) during seeding, dme-miRNA1-induced silencing was induced by cotransfection of pMT-*Renilla* luciferase (Rluc), pMT-Firefly luciferase (Fluc)-dme-miRNA1 sensor construct, either specific (dme-miRNA1) or off-target (dme-miRNA12) primary miRNA. The means of three independent experiments in duplicate are shown with the standard errors. Asterisks indicate significance determined by an independent two-sample Student *t* test ( $P < 0.05$ ). (C) Confirmation of successful Ago1/2 depletion in S2 cells. To check the knockdown level of Ago1/2, RT-PCR was carried out on total RNA purified from dsRNA-silenced cells. Western blot analysis of *Drosophila melanogaster* Ago1/2 was performed to confirm successful depletion of Ago1/2 in S2 cells.

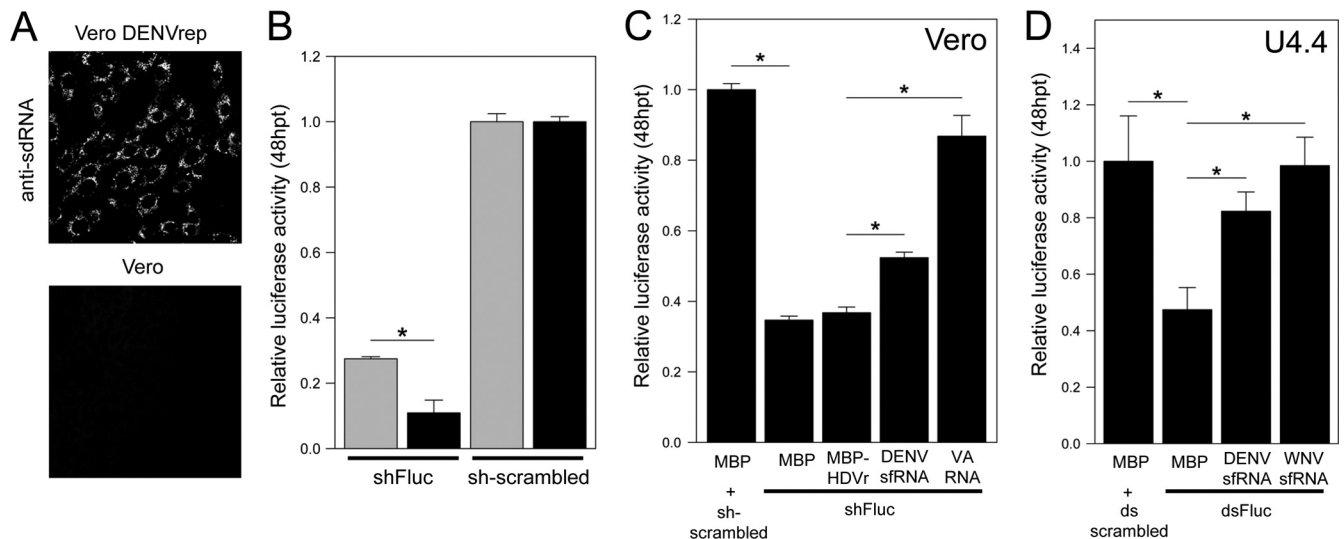
The RNA silencing pathway is the major antiviral response in mosquitoes against flavivirus infection (6), and the knockdown of this pathway results in an increase of viral replication in mosquitoes (55). It has remained unclear to what extent and how flaviviruses tweak this antiviral RNAi response for its own benefit. In the present study we now show that a noncoding, 3'UTR-derived viral RNA produced by the flaviviruses WNV and DENV displays RNAi suppressor activity in both insect and mammalian cells.

This noncoding viral RNA is present in the 3'UTR of all flavivirus genomes and, interestingly, is also abundantly produced as an XRN1-exonuclease-resistant molecule in flavivirus-infected cells of both insect and mammalian origin (52). This predominant noncoding RNA molecule was previously named subgenomic flavivirus RNA (sfRNA) and is completely identical to the 3'UTR with the exception that it misses the first ~100 nt (52). The flavivirus 3'UTR and hence also sfRNA share some structural similarities with the adenovirus VA RNAI/II molecules, which have been shown to act as efficient RSS in mammalian cells (3). Previously, it was shown that sfRNA is important for virus-induced cytopathic and pathogenicity in mammals (52), and more recent studies suggested the inhibition of the host innate immune response as one of the potential mechanisms of its action (59). Our present work suggests another potential mechanism for the sfRNA, i.e., as nucleic acid-based RSS both in both mammalian and in insect cells.

We have carried out most of the experiments with the plasmids expressing a truncated 3'UTR corresponding to the size of sfRNA (525 nt). Therefore, we cannot conclude at this stage whether or not the entire 3'UTR present in (the context of) the viral genome has the same RSS activity as sfRNA. Based on studies on genome cyclization during flavivirus infection, which show that the 3'UTR is base paired to the 5'UTR (26, 29, 67), the genomic viral 3'UTR may be far less accessible to RNAi factors compared to the abundantly produced sfRNA. The flaviviral genomic RNA replication is localized within endoplasmic reticulum membrane-enclosed vesicle packets (39, 73), whereas sfRNA is localized in cytoplasmic processing bodies (52), structures that are enriched in RNAi factors (16, 49). This suggests that sfRNA is more likely to interfere with RNAi than the 3'UTR of genomic viral RNA. Mutations in the 3'UTR leading to reduced expression of sfRNA (19, 52) also modify the secondary structure and likely function of sfRNA. This makes it difficult to discriminate whether the 3'UTR itself or the sfRNA, which is derived from it, displays the observed RSS activity in cells with active WNV replication.

Results from *in vitro* Dicer cleavage experiment suggest that WNV sfRNA may act as an RSS by substrate competition (Fig. 3), thereby possibly oversaturating Dicer in a concentration-dependent manner. Although this experiment is highly artificial and largely ignores the complexity of factors present in a flavivirus-





**FIG 8** Suppression of RNA silencing in mammalian and mosquito cells by DENV sfRNA. (A) Viral RNA replication in Vero cells stably expressing the DENV1 replicon was verified by immunofluorescence using a primary J2 anti-dsRNA antibody and a corresponding rhodamine-labeled secondary antibody (upper panel). Wild-type Vero cells were stained as a control (lower panel). (B) The effect of DENV RNA replication on shRNA-induced silencing was investigated in normal Vero cells (black) and in Vero cells stably expressing the DENV replicon (gray). The cells were cotransfected with Firefly luciferase (Fluc), *Renilla* luciferase (Rluc), and shRNA, either Fluc-specific (shFluc) or off-target (sh-scrambled). The normalized relative luciferase expression (Fluc/Rluc) was determined at 48 hpt. The means of three independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance determined by an independent two-sample Student *t* test ( $P < 0.05$ ). (C) RNA silencing suppression by the DENV sfRNA was investigated in Vero cells by cotransfection of pFluc, Rluc, either specific (shFluc) or off-target (sh-scrambled) shRNA, and expression constructs of MBP, MBP-HDVr, DENV, sfRNA, or adenoviral VA RNA. The luciferase expression was measured at 48 hpt. The means of two independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance by Tukey's HSD ( $P < 0.05$ ). (D) Effect of DENV sfRNA was determined by cotransfection of *Aedes albopictus*-derived U4.4 cells with Firefly luciferase (Fluc), *Renilla* luciferase (Rluc) and MBP, DENV sfRNA, or WNV sfRNA (as positive control) constructs. After 24 h, silencing was induced by transfection of either specific (dsFluc) or off-target (ds-scrambled) *in vitro*-transcribed dsRNA. The luciferase activity was determined 48 h after primary transfection and normalized to the cells transfected with off-target dsRNA. The means of three independent experiments performed in duplicate are shown with the standard errors. Asterisks indicate significance determined by Tukey's HSD ( $P < 0.05$ ).

infected cell, the proposed RNA decoy mechanism is supported by the observed concentration-dependent RNAi suppressor activity of sfRNA in shRNA-mediated silencing experiments (Fig. 3C and 6D), as well as the ability to bypass the RSS activity of sfRNA by transfection with siRNA (Fig. 3D). Nucleic acid-based decoy mechanisms in other viral systems have recently been published for SFV (61) and *Cauliflower mosaic virus* (CaMV) (7). In SFV infection, hot spot-derived viral siRNAs have been found to be rather inefficient in silencing of viral genomic RNA, thus allowing viral replication while saturating RNAi factors (61). During CaMV infection, high concentrations of RNA produced from a viral noncoding region are subsequently processed into siRNAs and incorporated into RISC to presumably act as a decoy (7). Although CaMV already encodes a RSS protein that interferes with the RNA-dependent RNA polymerase-mediated amplification of siRNA, the virus may in this case, paradoxically, benefit from the massive production of siRNAs derived from its noncoding RNA as backup strategy. Thus, abundant production of noncoding or viral siRNA can be advantageous for viral infection.

Although sfRNA itself may not lead to massive production of decoy viral siRNA, its strong secondary structure (19, 45, 52) may decrease overall Dicer activity or render the genomic viral RNA inaccessible for an activated RISC. The suggested RNA decoy mechanism by sfRNA probably results in a lower RSS activity compared to that of most of the known proteinaceous RSS of true insect viruses but may still allow sufficient levels of flaviviral replication needed for successful virus transmission by the vector mosquitoes. Recent research has shown that the expression of

protein RSS (derived from true insect viruses) by alphaviruses leads to the rapid death of infected mosquitoes (11, 43). Similarly, we now show that expression in *cis* of sfRNA also enhances SFV replication in RNAi-competent mosquito cells (Fig. 6B). In light of these results, it is clear that a delicate balance exists between arbovirus replication and vector survival. Although all arboviruses for which this has been analyzed induce RNAi responses, it now appears that inhibition or evasion does take place (61), and it is likely that the scale of inhibition or evasion is a key factor. Our present results do not allow us to directly compare RNAi suppressor activity of sfRNA to that of the protein RSS, but future work will focus on addressing this important question. It is worth noting, however, that the level of sfRNA produced from plasmid DNA transfection in most of our RNAi suppressor assays is lower than the sfRNA level produced in virus-infected or replicon-transfected cells (52).

Despite the fact that all flaviviruses produce sfRNA (52) and thus might suppress RNAi, flavivirus replication can still be inhibited by endogenous miRNA when miRNA target sites are artificially engineered in the viral RNA (23, 30, 51). This suggests that sfRNA does not fully block RNAi, which is in agreement with the lower RSS activity of sfRNA in comparison to, for example, VA RNA.

In addition to the RSS activity of sfRNA on the antiviral siRNA pathway in insect cells, we observed similar suppressor activity of sfRNA on the insect miRNA pathway. In line with this result, others have recently shown that WNV infection of *Culex* mosquitoes alters the expression levels of a subset of host miRNAs (62). In

contrast, *Aedes albopictus* C7/10 mosquito cells harboring a WNV replicon did not display these changes in endogenous miRNA expression (62). However, the relative levels of viral RNA replication, which normally correlate with sfRNA expression levels, of persistent WNV replicon in C7/10 versus virus infection of *Culex* mosquitoes was not shown. It remains therefore possible that sfRNA levels in C7/10 were too low to observe an effect on the miRNA profile or that defects in RNAi factors in C7/10 cells (42) play a determining role.

Dual activity of RSS proteins on siRNA- and miRNA-related pathways has been observed previously for plant-infecting viruses such as *Tomato spotted wilt virus* (58) and tombusvirus (66), as well as the adenoviral VA RNA (38), but their functional importance during viral replication is still unclear. In insects no such mechanisms have been reported yet, but some results suggest the importance of other antiviral responses in addition to the siRNA pathway. For example, a negative effect on viral replication of WNV lacking sfRNA has been observed in Dicer-2-deficient C6/36 mosquito cells (52). This could be due to the activity of Dicer-1 (miRNA-related) or a Dicer-independent RNA silencing pathway involving piRNAs (42, 68) or to another antiviral response independent of RNAi (17).

Herpes-, adeno-, baculo-, and ascoviruses produce virus-encoded miRNAs targeting either viral RNA or host genes involved in cell proliferation, DNA repair, and RNA regulation (5, 20). Adenoviral VA RNAs are processed by Dicer to viral miRNAs that are loaded into Argonaute, which can result in its saturation. In light of the similarities between the secondary structures of adenovirus VA RNA and the WNV sfRNA, it may be possible that the interaction of sfRNA with the miRNA pathway is related to the processing of a virus-encoded miRNA. Indeed, sfRNA was recently shown to be the main source of 3'SL-derived viral miRNA in WNV-infected insect cells (25). It is therefore possible that the functionality of sfRNA as Dicer substrate may determine its RSS activity, but our results with miRNA sensors show that this viral miRNA is not (efficiently) produced in mammalian cells (Fig. 5D). In addition, sfRNA variant lacking the 3'SL still did display some RSS activity (Fig. 5F), suggesting that production of 3'SL-derived viral miRNA is likely not required for sfRNA to interfere with the RNAi machinery. Thus, despite the similarities with VA RNA, which is very efficiently loaded into RISC as viral miRNA (75), sfRNA displays RSS activity in the cytoplasm without being fully processed. To fully understand the biological importance of RNAi suppression by sfRNA in mammalian cells, more elaborate studies (e.g., on the effect of the depletion of Dicer or other RNAi components) on flavivirus replication would be needed.

There is some evidence that might suggest the involvement of miRNA in antiviral RNAi in mammalian cells (for a review, see reference 47) and possible links between the miRNA pathway and the IFN response (50, 74). A dual function of a viral product in both of these pathways has been reported for adenovirus VA RNA, which is able to interact with both the IFN response and the RNAi in mammalian cells. We previously reported that sfRNA was required for virus-induced pathogenicity in mammals and hypothesized a putative function in the antiviral IFN pathway (52). Indeed, recent data show that sfRNA contributes to viral evasion of the type I IFN-mediated antiviral response (59). It is therefore tempting to speculate that the observed effects of sfRNA on the RNAi pathways and on modulation of the IFN response in mam-

malian cells are linked, although further studies are required to support this supposition.

In conclusion, our findings show that sfRNAs of WNV and DENV can interfere with two distinct arms of the RNAi pathway—siRNA and miRNA mediated—in both insect and mammalian cells. Future experiments will elucidate whether this novel sfRNA function holds for all flaviviruses and will hopefully reveal the complete picture describing the different functional roles of flavivirus noncoding RNA in the innate immune responses of mosquitoes and vertebrate hosts.

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